Supplemental Material

Genotoxic Effects in Swimmers Exposed to Disinfection By-products in Indoor Swimming Pools

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Comet analysis in peripheral blood lymphocytes. The comet assay was performed as described previously (Singh et al. 1988) with minor modifications. Blood samples were collected in vacutainers with EDTA. Samples were kept chilled, and the length of time between blood collection and sample processing was a few hours (Anderson et al 1997). A drop (7 µL) of whole blood was resuspended in 75 µL of 0.5% low-melting-point agarose, layered onto microscope slides pre-coated with 150 µL of 0.5% normal-melting-point agarose (dried at 65ºC), covered with a coverslip, and kept at 4ºC until solidified. Then the coverslips were removed, and the cells were lysed overnight at 4ºC in a dark chamber containing fresh, cold lysing solution (2.5-M NaCl, 100-mM Na_2EDTA, 10-mM Tris, 10% DMSO, 1% Triton X-100, and 1% laurosylsarcosinate, pH 10). To allow DNA denaturation, unwinding, and exposure of alkali-labile sites, slides were placed for 20 min in a horizontal gel electrophoresis tank filled with fresh, cold electrophoresis solution (1-mM Na_2EDTA and 300-mM NaOH, pH 13.5). Electrophoresis was performed in the same buffer for 20 min at 0.73 V/cm and 300 mA. Unwinding and electrophoresis steps were done in an ice bath.

After electrophoresis, slides were neutralized with 2, 5-min washes with 0.4-mM Tris (pH 7.5), fixed with absolute ethanol for 3 min, and stored in the dark at room temperature until scoring. Just before microscopic analysis, slides were stained with 60 µL of ethidium bromide (0.4 µg/mL). The images were examined at 400X magnification with a Komet 5.5 Image Analysis (Kinetic Imaging Ltd, Liverpool, UK) fitted with a Olympus BX50 fluorescent microscope equipped with a 480-550-nm wide band excitation filter and a 590-nm barrier filter. One hundred cells selected randomly (50 cells from each of the two replicate slides) were analyzed per sample. Olive tail moment (OTM) and percentage of DNA in the tail were used as measures of DNA damage and computed using Komet Version 5.5 software.

MN analysis in peripheral blood lymphocytes. Blood was obtained from each subject by venipuncture using heparinized vacutainers and sent immediately to the laboratory for the lymphocyte cultures. Lymphocyte cultures were set up by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium supplemented with 15% heat-inactivated fetal calf serum, 50 U/mL penicillin, 50 µg/mL streptomycin, and 2-mM L-glutamine (all provided by PAA Laboratories GmbH, Pasching, Austria). Lymphocytes were stimulated by addition of 1% phytohaemagglutinin (Gibco, Life Technologies, Paisley, UK) and incubated for 72 h at 37°C. Two replicate cultures were prepared for each blood sample. Cytochalasin B (Cyt-B) (Sigma, St Louis, MO) at a final concentration of 6 µg/mL (Surrallés et al. 1994) was added to the cultures after a 44-h incubation to arrest cytokinesis. At 72 h of incubation, the cultures were harvested by centrifugation at 800 rpm for 8 min. Next, in order to eliminate red blood cells and to preserve cytoplasm, the cell pellet was treated with a hypotonic solution (7 min in 0.075-M KCl at 4°C). Cells were then centrifuged, and a methanol/acetic acid (3:1 v/v) solution was added gently. This fixation step was repeated twice, and the resulting cells were re-suspended in a small volume of fixative solution and dropped onto clean slides. Finally, they were stained with 10% Giemsa (Merck, Darmstadt, Germany) in phosphate buffer, pH 6.8, for 7 min.

To determine the frequency of binucleated cells with micronuclei (BNMN) and the total number of micronuclei, a total of 1000 binucleated cells with well-preserved cytoplasm (500 per replicate) were scored for each subject. In addition, 500 lymphocytes were scored to evaluate the percentage of cells with one to four nuclei, and the cytokinesis block proliferation index (CBPI) was calculated (Surrallés et al. 1995). Microscopic scoring was performed on coded slides.

MN analysis in urothelial cells. Urine samples were collected before swimming and again 2 weeks later in plastic vials (~50 mL) and sent to the laboratory where they were processed the same day. Two weeks was selected because this amount of time is required for exfoliation of cells from the urothelium (Espinoza et al. 2008). Cell samples were concentrated by centrifugation (10 min at 1500 rpm), the supernatant was discarded, and cells were washed in a NaCl solution (0.9%). Cells were centrifuged again and re-suspended in a new NaCl solution (0.09%). After another centrifugation, cells were fixed in 5 mL of a fresh fixative solution (methanol/acetic acid, 3:1), added drop by drop. After 1-2 washings with the fixative solution, the pellet was re-suspended in 1 mL of fixative solution and dropped onto pre-cleaned microscope slides. Cell density was confirmed by using a phase-contrast microscope (400 x) and adjusted by adding fixative solution. Slides were air dried overnight and stored at 4ºC in the dark until staining. Cells were stained with the DNA-specific stain 4',6-diamino-2-phenylindole dihydrochloride (DAPI) at 1 μg/mL, which avoids possible scoring artifacts. One scorer using an Olympus BX50 fluorescence microscope (1000 x) scored a total of 2000 cells/donor, wherever possible, on coded slides.

The criteria for MN evaluation were those suggested by Stick et al. (1983) as updated by subsequent guidelines by Fenech et al (2003). The frequency of cells with MN and the total number of MN were determined for each analyzed subject. Only those cells with a typical morphology corresponding to the urothelial cells were scored. This criterion avoids any kind of bias, especially in women where many squamous cells not of urothelial origin are observed. Although bacteria were present in a few urine samples, they did not interfere with the scoring.

Urine mutagenicity. Urine samples (30 mL) were collected prior to and 90 to 120 min after exposure and were evaluated for mutagenicity in the *Salmonella* (Ames)

mutagenicity plate-incorporation assay (Maron and Ames 1983) in strain YG1024 with S9 mix. YG1024 is a frameshift strain derived from TA98 (*hisD3052, ΔuvrB, rfa,* pKM101) that contains acetyltransferase activity (Watanabe et al. 1990); it has been used extensively for urinary mutagenicity studies (Cerná and Pastorková 2002). Only one strain was used because of the limited availability of sample.

To extract urinary organics, 25 ml of urine was passed through C18 resin, and the organics were eluted by methanol and then solvent exchanged into dimethyl sulfoxide (DMSO) at 150x for bioassay as described (DeMarini et al. 1997). Extracts were evaluated once in single plates per dose at 0.3, 0.6, 1.2, 3, 6, and 12 mL-equivalents per plate. Controls consisted of DMSO (100 μl per plate), C18 resin blanks prepared by passing 40 mL of distilled deionized water instead of urine through the columns (15 mL equivalent per plate), and 2-acetylaminiofluorene at 1 μg per plate (positive control). Control values (revertants per plate) were 9–14 (DMSO), 5–10 (resin blanks), and 377–595 (2-acetylaminofluorene). The mutagenic potencies of samples, expressed as revertants (rev) per mL-equivalent, were calculated from the slope of the regression over the linear portion of the dose-response curves. Slopes were calculated for only 43 subjects with samples from before and after swimming that were sufficient for analyses of mutagenicity at ≥3 different concentrations

Gene selection and genotyping. Blood was collected in EDTA tubes and stored in the same tube at -80^{o}C for DNA extraction. DNA was extracted using the Chemagic Magnetic Separator Technology (Chemagen) at the Spanish National Genotyping Centre (CEGEN) and quantified using PicoGreen dsDNA fluorescent detection system (PicoGreen, Molecular Probes). We examined genetic variants, including single-nucleotide polymorphisms (SNPs), and copy-number variants (CNVs) in three genes involved in the metabolism of DBPs (*GSTT1, CYP2E1,* and *GSTZ1),* four additional

genes that may play a minor role in the metabolism of DBPs (*GSTT2B, GSTM1, CYP1A2* and *CYP2D6*), and four DNA repair genes that could be relevant when examining results, particularly for the comet assay (*APEX1, ERCC2, OGG1, XRCC1*). We selected tagSNPs combined with functional variants most likely to influence gene expression or function. In total, three CNVs and 17 SNPs were genotyped. For a complete list, see Supplemental Material, Tables 1-4.

SNP genotyping was performed at the Santiago de Compostela node of the "Centro Nacional de Genotipado" (CEGEN) in Spain (http://www.cegen.org) using the Sequenom® platform. Individuals with low genotyping frequency (<75%) or non-Caucasians were excluded from the genetic analyses (n = 4). Genotyping failed for the following SNPs: rs11101815 and rs915908 in *CYP2E1*, rs1799793 in *ERCC2*, and rs28903081 in the *XRCC3* gene. The remaining 13 SNPs had a call frequency >90%. Genotyping quality was controlled by using positive controls consisting of one HapMap reference trio. CNVs for *GSTT1, GSTT2B,* and *GSTM1* were genotyped as described previously with minor modifications (Buchard et al. 2007; Zhao et al. 2009). Briefly, the genotyping consisted of multiplex PCR amplifications, where deleted and non-deleted alleles gave different fragment sizes that were resolved in agarose gels. The CNV call frequency was >90%. Genotyping was repeated for selected samples and gave consistent genotypes.

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Supplemental Material, Table 1. Interaction between the effect of exposure to bromoform on micronuclei in peripheral blood lymphocytes in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number Subjects	Beta-coefficient	95%CI		*p*-value for each genotype [a]	*p*-value interaction [a]
GSTT1	-/-	16	7.773	1.90	13.65	0.014	0.367
	-/+, +/+	30	3.389	-1.55	8.33	0.170	
GSTT2B	-/-, -/+	35	1.595	-2.32	5.51	0.412	0.016
	+/+	11	9.608	-0.11	19.33	0.052	
GSTM1	-/-	22	5.401	-0.67	11.47	0.078	0.892
	-/+, +/+	24	5.075	-0.94	11.09	0.094	
CYP2E1	TT	27	4.349	-0.41	0.91	0.071	0.447
rs2070673	AT_AA	16	-0.027	-7.52	7.46	0.994	
CYP2E1	TT	28	6.652	2.24	11.06	0.005	0.143
rs915906	CT/CC	14	-5.910	-14.11	2.29	0.139	
CYP2E1	CC	30	3.676	-1.78	9.13	0.178	0.655
rs915907	CA_AA	14	6.554	-1.98	15.09	0.118	
CYP2E1	CC	35	5.758	1.66	9.85	0.007	0.907
rs2515641	CT	9	-0.363	-14.07	13.34	0.948	
CYP2E1	CC	25	4.215	-0.81	9.24	0.096	0.381
rs2249695	CT_TT	19	7.448	1.09	13.80	0.025	
GSTZ1	GG	22	10.155	2.98	17.32	0.008	0.039
rs3177427	AG_AA	22	1.384	-3.73	6.50	0.577	
GSTZ1	CC	29	3.256	-0.96	7.48	0.125	0.733
rs1046428	CT_TT	14	-5.371	-18.49	7.75	0.383	
CYP1A2	AA	20	6.288	2.61	9.97	0.002	0.886
rs762551	AC_CC	21	2.899	-5.61	11.41	0.482	
CYP2D6	GG	32	4.958	0.74	9.17	0.023	0.558
rs3892097	AG	11	7.111	-7.59	21.81	0.290	
APEX1	TT	17	9.456	3.30	15.61	0.006	0.016
rs11304009	GT_GG	27	-2.457	-9.39	4.47	0.471	
ERCC2	TT	17	9.999	2.02	17.97	0.018	0.092
rs13181	GT_GG	27	2.660	-1.38	6.70	0.186	
OGG1	CC	28	5.760	1.14	10.37	0.017	0.727
rs1052133	CG/GG	16	2.608	-7.36	12.57	0.579	
XRCC1	GG	16	4.531	-1.77	10.83	0.143	0.674
rs25487	AG_AA	28	6.181	1.04	11.32	0.020	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplemental Material, Table 2. Interaction between the effect of exposure to bromoform on micronuclei in exfoliated cells from urine in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype [a]	*p*-value interaction [a]
GSTT1	-/-	10	-5.189	-48.63	38.25	0.780	0.699
	-/+_+/+	23	1.732	-11.14	14.61	0.781	
GSTT2B	-/-_-/+	26	-0.054	-12.45	12.34	0.993	0.387
	+/+	7	-1.419	-59.90	57.06	0.943	
GSTM1	-/-	12	-23.145	-50.45	4.16	0.086	0.004
	-/+_+/+	21	8.108	-2.07	18.29	0.111	
CYP2E1	TT	21	10.546	0.90	20.19	0.034	0.068
rs2070673	AT_AA	10	-11.492	-41.07	18.09	0.378	
CYP2E1	TT	22	11.618	2.45	20.79	0.016	0.021
rs915906	CT/CC	9	-23.161	-52.44	6.11	0.098	
CYP2E1	CC	22	12.107	-1.32	25.53	0.074	0.366
rs915907	CA_AA	9	9.762	-12.32	31.85	0.321	
CYP2E1	CC	25	7.584	-3.21	18.38	0.159	0.842
rs2515641	CT	6	-10.963	-23.57	1.64	0.065	
CYP2E1	CC	21	10.546	0.90	20.19	0.034	0.068
rs2249695	CT_TT	10	-11.492	-41.07	18.09	0.378	
GSTZ1	GG	19	-0.176	-19.36	19.01	0.985	0.521
rs3177427	AG_AA	12	8.657	-5.62	22.93	0.200	
GSTZ1	CC	20	5.788	-5.65	17.22	0.299	0.138
rs1046428	CT_TT	11	21.132	-7.55	49.82	0.125	
CYP1A2	AA	15	6.238	-8.45	20.93	0.373	0.452
rs762551	AC_CC	16	13.721	-5.57	33.01	0.147	
CYP2D6	GG	23	11.135	0.44	21.83	0.042	0.394
rs3892097	AG	7	-5.176	-48.69	38.34	0.730	
APEX1	TT	10	6.778	-4.64	18.19	0.197	0.317
rs11304009	GT_GG	21	1.555	-19.82	22.93	0.880	
ERCC2	TT	13	1.693	-26.50	29.89	0.895	0.146
rs13181	GT_GG	18	12.883	3.48	22.29	0.011	
OGG1	CC	20	4.112	-7.17	15.40	0.451	0.095
rs1052133	CG/GG	11	29.044	-3.13	61.21	0.070	
XRCC1	GG	14	14.641	4.66	24.62	0.008	0.157
rs25487	AG_AA	17	-5.626	-25.28	14.03	0.547	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplemental Material, Table 3. Interaction between the effect of exposure to bromoform on urine mutagenicity in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype[a]	*p*-value interaction[a]
GSTT1	-/-	14	1.902	-4.15	7.96	0.500	0.112
	-/+_+/+	26	7.590	2.97	12.21	0.003	
GSTT2B	-/-_-/+	30	6.296	1.34	11.25	0.015	0.410
	+/+	10	2.806	-3.19	8.81	0.296	
GSTM1	-/-	18	6.066	0.92	11.21	0.024	0.734
	-/+_+/+	22	4.679	-1.58	10.94	0.134	
CYP2E1	TT	22	6.893	0.74	13.04	0.030	0.341
rs2070673	AT_AA	15	3.010	-2.93	8.95	0.288	
CYP2E1	TT	22	5.844	1.83	9.86	0.007	0.381
rs915906	CT/CC	14	3.142	-3.95	10.23	0.347	
CYP2E1	CC	27	4.865	-0.85	10.58	0.091	0.986
rs915907	CA_AA	11	5.375	-5.07	15.82	0.263	
CYP2E1	CC	29	5.529	1.10	9.96	0.017	0.609
rs2515641	CT	9	2.881	-9.74	15.51	0.583	
CYP2E1	CC	21	7.332	2.78	11.88	0.003	0.083
rs2249695	CT_TT	17	2.457	-2.71	7.62	0.323	
GSTZ1	GG	21	3.478	-1.78	8.74	0.181	0.213
rs3177427	AG_AA	17	8.697	2.02	15.37	0.015	
GSTZ1	CC	24	6.523	1.86	11.18	0.009	0.708
rs1046428	CT_TT	13	-2.092	-18.87	14.68	0.784	
CYP1A2	AA	17	5.183	-2.48	12.84	0.168	0.274
rs762551	AC_CC	19	10.293	3.04	17.54	0.009	
CYP2D6	GG	26	4.800	1.40	8.20	0.008	0.810
rs3892097	AG	11	3.342	-9.75	16.44	0.565	
APEX1	TT	15	4.709	-3.14	12.55	0.213	0.543
rs11304009	GT_GG	23	0.447	-4.44	5.34	0.850	
ERCC2	TT	16	2.253	-4.05	8.55	0.451	0.121
rs13181	GT_GG	22	8.454	3.04	13.87	0.004	
OGG1	CC	24	5.563	0.68	10.44	0.028	0.630
rs1052133	CG/GG	14	0.473	-4.72	5.67	0.843	
XRCC1	GG	15	8.835	3.62	14.05	0.003	0.073
rs25487	AG_AA	23	2.431	-2.89	7.76	0.351	

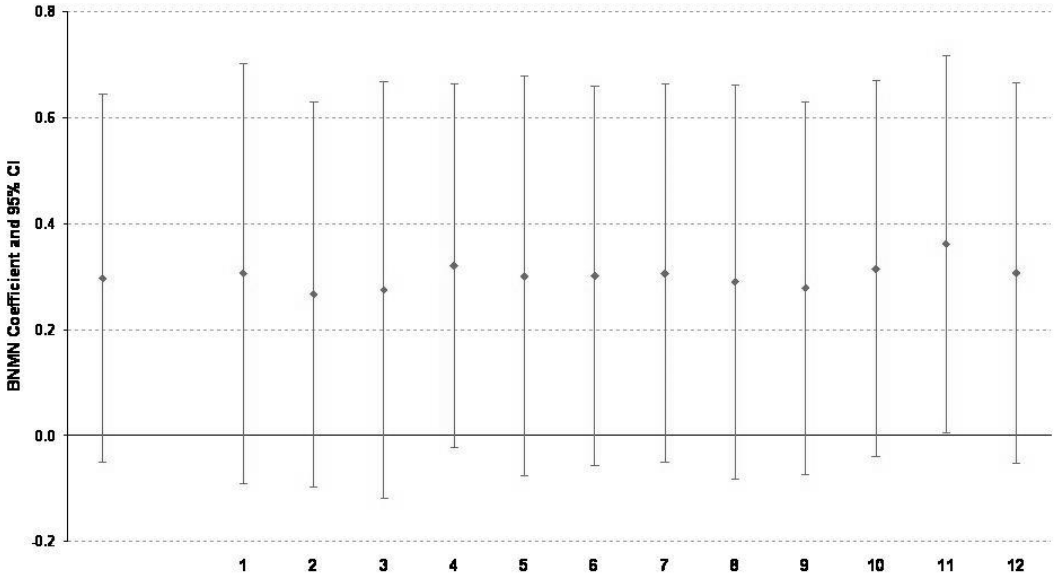
[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplemental Material, Table 4. Interaction between the effect of exposure to bromoform on the comet assay in relation to polymorphisms in genes associated with metabolism of DBPs or with DNA repair

Gene	Genotypes	Number subjects	Beta-coefficient	95%CI		*p*-value for each genotype[a]	*p*-value interaction[a]
GSTT1	-/-	16	-0.043	-0.81	0.72	0.905	0.468
	-/+_+/+	30	-0.433	-1.25	0.38	0.285	
GSTT2B	-/-_-/+	35	-0.402	-1.18	0.37	0.299	0.437
	+/+	11	0.117	-0.71	0.94	0.748	
GSTM1	-/-	22	0.312	-0.64	1.27	0.500	0.065
	-/+_+/+	24	-0.629	-1.35	0.96	0.085	
CYP2E1	TT	27	-0.308	-1.10	0.49	0.430	0.347
rs2070673	AT_AA	16	0.048	-1.34	1.44	0.941	
CYP2E1	TT	28	-0.394	-1.03	0.24	0.215	0.330
rs915906	CT/CC	14	-0.108	-2.24	2.02	0.912	
CYP2E1	CC	30	0.391	-0.50	1.28	0.374	0.191
rs915907	CA_AA	14	-0.846	-1.65	-0.04	0.042	
CYP2E1	CC	35	-0.303	-0.94	0.34	0.343	0.707
rs2515641	CT	9	0.781	-1.96	3.52	0.497	
CYP2E1	CC	25	-0.305	-1.10	0.49	0.436	0.613
rs2249695	CT_TT	19	-0.124	-1.08	0.83	0.786	
GSTZ1	GG	22	-0.227	-1.16	0.71	0.617	0.637
rs3177427	AG_AA	22	-0.496	-1.48	0.49	0.303	
GSTZ1	CC	29	-0.348	-1.11	0.41	0.357	0.420
rs1046428	CT_TT	14	0.668	-1.85	3.19	0.567	
CYP1A2	AA	20	-0.513	-1.43	0.41	0.256	0.139
rs762551	AC_CC	21	0.753	-0.62	2.13	0.264	
CYP2D6	GG	32	-0.515	-1.12	0.09	0.094	0.062
rs3892097	AG	11	1.251	0.07	2.43	0.041	
APEX1	TT	17	-0.348	-1.32	0.62	0.452	0.210
rs11304009	GT_GG	27	0.353	-0.77	1.48	0.522	
ERCC2	TT	17	0.241	-0.73	1.21	0.601	0.378
rs13181	GT_GG	27	-0.295	-1.02	0.43	0.408	
OGG1	CC	28	-0.159	-0.79	0.47	0.605	0.394
rs1052133	CG/GG	16	-1.058	-3.04	0.92	0.267	
XRCC1	GG	16	-0.573	-1.51	0.37	0.209	0.171
rs25487	AG_AA	28	0.094	-0.65	0.84	0.797	

[a]The *p*-value for each stratum evaluates statistical significance of the effect of exposure among subjects with the given genotype. The *p*-value for interaction evaluates whether the effect of exposure differs between two genotypes or grouped genotypes.

Supplemental Material, Figure 1. Association between total THMs in exhaled breath and changes in the formation of micronuclei when adjusting for age and sex (main model) or additionally adjusting for 12 other variables (n = 49 subjects)[a].



[a]All models include age and sex. Models 1 to 12 include also the following variables: Model 1 water consumption, 2 source of water, 3 antioxidant intake from diet, 4 swimming at least once per month, 5 number of laps during experiment (an indication of physical activity), 6 leisure physical activity at least once per week, 7 self-reported work exposure to chemical, physical of biological agents, 8 exposure to second-hand smoke, 9 use of permanent hair dyes, 10 shower before the experiment, 11 leisure time physical activity last 24 h, 12 vitamins last 24 h.